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Response of *Neisseria gonorrhoeae* to *Bdellovibrio* Species

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Bdellovibrio species are small, highly motile bacteria that are predators upon other bacteria in nature. Bdellovibrios attach to, penetrate, replicate within, and destroy prey that share the general characteristic of gram negativity. The lipopolysaccharide moiety of the cell membrane of target microorganisms appears to contain the principal receptor site for bdellovibrio attachment. Since gonococci also contain lipopolysaccharide that is similar in many respects to that contained within gram-negative rods, studies were conducted to determine the extent of gonococcal interaction with a variety of *Bdellovibrio* species. Despite transient attachment, penetration of gonococci by bdellovibrios never occurred. Failure of bdellovibrio parasitization was unrelated to gonococcal species, colony type, piliation, penicillin susceptibility, or virulence as influenced by passage in embryonated eggs. In experiments involving mixtures of gonococci and more typical gram-negative bacillary prey, the latter were always attacked by bdellovibrios, whereas the former were ignored. Despite evidence for similarities between gonococcal and gram-negative bacillary lipopolysaccharides, resemblances do not extend to the point where gonococci are susceptible to bdellovibrio parasitization.

Bdellovibrio species are ubiquitous aerobic gram-negative bacteria, extremely small, that attack and infect other bacteria by growing in the space between their cell wall and cell membrane (13). The relationship is more accurately that of predator-prey than parasite-host (2). After an often violent, and perhaps chemotactically mediated, collision between rapidly moving bdellovibrios and their targets (15), attachment occurs via several pilus-like filaments (13, 15). The bdellovibrios then bore and/or enzymatically digest their way into the periplasm of their prey where they degrade target bacterial cell components, utilizing them for growth and energy (15). Infected cells often swell as their ability to maintain osmotic integrity is lost. These forms have been referred to as "spheroplasts" (13) or "bdelloplasts" (14). After a period of elongation and daughter cell formation, mature bdellovibrios are released from disintegrating target bacteria within a few hours of the initial attack (13, 15).

Bacteria that serve as prey to *Bdellovibrio* species share the general characteristic of gram negativity. Bdellovibrios are variably prey specific. Sufficient *Bdellovibrio* species have been characterized that a number of diverse gram-negative bacilli are included in their cumulative range of activity. Among these are *Escherichia coli, Salmonella* sp., *Proteus mirabilis, Pseudomonas putida, Serratia marcescens,* and others (13, 15, 16). Bdellovibrios apparently "home" upon receptors within the cell wall of target bacteria. Rough strains of *Salmonella* sp. and *E. coli* (especially chemotype Ra) lacking O-specific antigens are somewhat more susceptible to attack than wild-type smooth strains in which O antigens appear to transiently retard access to receptor sites (19). Further evidence that R antigen contains the receptor site for bdellovibrio attachment is suggested by the observation that extracted *Salmonella typhimurium* R antigen blocks interaction of *Bdellovibrio bacteriovorus* with this microorganism (3).

Gonococci contain lipopolysaccharide in their cell walls and are very similar in this respect to gram-negative bacilli (6). The present study was designed to determine whether diverse *Bdellovibrio* species would attach to and invade gonococci, and whether such interaction would be a function of gonococcal strain, colony type (piliation), penicillin sensitivity (9), or virulence (agar plate versus egg passaged [20]).

MATERIALS AND METHODS

Bdellovibrio species. Five bdellovibrios were employed. *B. bacteriovorus* 109J was obtained from S. C. Rittenberg of the Department of Bacteriology, University of California at Los Angeles. This microorganism was maintained in cultures of *E. coli* ML-35 as described (12). Four additional bdellovibrios, together with their definitive target microorganisms, were obtained from the American Type Culture Collection (ATCC), Rockville, Md. These included *B. bacteriovorus* 110 (ATCC 15360) and *Enter-*

obacter cloacae (ATCC 15361), *B. bacteriovorus* 114 (ATCC 15362) and *P. mirabilis* (ATCC 15363), *B. bacteriovorus* 118 (ATCC 15364) and *S. marcescens* (ATCC 15365), and *B. starrii* A3.12 (ATCC 14145) and *P. putida* (ATCC 12633). The ATCC strains and their target bacteria were received in lyophilized form and were reconstituted and passaged together serially for 1 to 2 weeks before being used in experiments. Bdellovibrios for individual experiments were grown overnight in cultures of their respective prey at 30 C on a shaking incubator as described (12, 18). Within 18 to 24 h, few intact prey remained. Bdellovibrios were harvested by differential filtration with a 1.2-μm filter (Millipore Corp., Bedford, Mass.) that retained intact target bacilli and bdelloplasts while allowing free bdellovibrios to pass (18).

Gonococci. Eleven strains of *Neisseria gonorrhoeae* were employed including strain F-62 (from Frank Young, Department of Microbiology; University of Rochester School of Medicine and Dentistry, New York); strain NS, a clinical isolate used extensively in studies in our laboratory; and nine clinical isolates (strains 1196, 1252, 1256, 1295, 1296, 1360, 1446, 1601, and Church) from the University of Texas-Bexar County Hospital clinical laboratories. All microorganisms were identified as gonococci by typical colony morphology on Thayer-Martin medium; oxidase positivity; fermentation of glucose, but not maltose, sucrose, or lactose; and labeling with fluorescent antibody (10). Presence of piliation was confirmed by electron microscopy (17). Colony morphology was maintained by selective subculturing; colony types were identified according to standard criteria (5). The minimal inhibitory concentration of penicillin for the F-62 strain was 0.04 μg/ml and for the NS strain, 0.4 μg/ml by plate-dilution techniques (11).

All gonococci for experiments were grown in T-1 colony morphology on gonococcal agar base (BBL, Becton-Dickenson and Co., Cockeysville, Md.) supplemented with Isovitalex (Fisher Scientific Co., Pittsburgh, Pa.) in the absence of antibiotics and incubated at 36 C in a CO_2 incubator or candle-extinction jar. F-62 gonococci were also grown in T-3, and NS was grown in T-2 and T-3 colony morphology. Microorganisms were harvested after 16 to 18 h of growth by gentle scraping with a wire loop and transferred to one of the media noted below in a final concentration of 10^7 to 10^9 gonococcal colony-forming units/ml.

For all experiments involving F-62 and NS strains, T-1 and T-3 gonococci were also passaged serially in the allantoic cavity of 10-day-old embryonated eggs (Texas A and M University, College Station, Tex.) to ensure maintenance of virulence, as described (20). Gonococci were then subcultured on gonococcal agar with Isovitalex for one single passage before being harvested and used in an experiment.

Other bacteria. Additional bacteria involved in some experiments included *Staphylococcus aureus* strain 502A, *Listeria monocytogenes* strain UCLM-1, and a wild-type *E. coli* (designated UTEC-1).

Interaction of bdellovibrios and target bacteria. Bdellovibrios are susceptible to damage in salt-containing solutions (18). Bdellovibrio-prey interactions are also best observed in media that fail to support optimal multiplication of the prey (13, 16). Therefore all studies were carried out in dilute nutrient broth (18) and/or tris(hydromethyl)amino- methane yeast extract-peptone medium broth medium 137, ATCC) (16). After 90 min in these media, gonococci became more spherical, tended to clump, and often assumed a ghost-like appearance. Since bdellovibrio attachment to susceptible target microorganisms occurs very rapidly, and since bdellovibrios will attack microorganisms that are either living or dead (13), gonococcal swelling was not judged to be a serious problem in this regard.

Bdellovibrios were mixed with gonococci at 30 or 37 C on a shaking water bath in ratios ranging from 1:1 to 100:1 in a total volume of 3 to 5 ml of medium. The progress of bacterial interaction in aliquots of medium was observed at regular intervals under phase microscopy employing a Zeiss Photomicroscope III and a magnification of $\times$2,000. In quantifying gonococcal-bdellovibrio interactions, a minimum of 200 individually discernible gonococcal pairs was evaluated; gonococcal clumps were avoided. Nevertheless, the periphery of gonococcal clumps was always carefully examined for evidence of bdellovibrio attachment. Simultaneous controls included bdellovibrios alone, gonococci alone, and bdellovibrios with their respective normal host bacilli. In some experiments, gonococci and the normal host bacilli (or the nonhosts *S. aureus* 502A and *L. monocytogenes*) were mixed in the same system to allow comparison of bdellovibrio selectivity for each potential target microorganism.

RESULTS

Failure of interaction was observed between gonococci and bdellovibrios, as indicated in Table 1. Whereas collisions of some violence occurred between bdellovibrios and their normal bacillary prey, rarely were there impacts of any magnitude between bdellovibrios and gonococci. A frequent observation was the prolonged maneuvering of isolated bdellovibrios around individual gonococci, as if searching for a place to attack. After a period of time, the bdellovibrio would seem to lose interest and move on, although on some occasions bdellovibrios were seen to establish initial contact with gonococcal pairs at the point of central constriction of the diplococci (Fig. 1). Vigorous motion of the "arm-in-socket" type, however, was rarely observed and penetration never occurred (13). When gonococci were mixed with more natural target bacilli for individual bdellovibrios, there was rapid attachment and penetration of the gram-negative rods by the bdellovibrios, whereas gonococci (and *S. aureus* 502A and *L. monocytogenes*) were ignored. Failure of bdellovibrio-gonococcal interaction occurred regardless of gonococcal colony morphology (T1, T2, or T3), piliation (T1), penicillin susceptibility, or mo-

TABLE 1. *Interaction of Bdellovibrio species with various host microorganisms*

Bdellovibrio species	Bacterial host	No. of experiments[a]	Time (min)			
			0	30	60	90–120
109J	*E. coli* ML-35	6	1–11/1-2[b]	14–22/8–12	9–13/16–31	3–9/17–26
	E. coli UTEC-1	6	2–4/0–3	10–17/5–8	4–16/15–33	7–9/16–23
	P. mirabilis	1				
	S. aureus 502A	2				
	L. monocytogenes	1				
	GC-F62[c]	2				
	GC-NS[d]	2				
	GC-miscellaneous (9) (T-1)	2				
110	*E. cloacae*	2	2–3/1	6–7/4–5	9–11/18–23	6–7/67–74
	E. coli ML-35	2				
	GC-F62[c]	2				
	GC-NS[d]	2				
114	*P. mirabilis*	2	47–51/1–3	73–81/1	76–85/0–5	81–96/3–8
	E. coli ML-35	2				
	GC-F62[c]	2				
	GC-NS[d]	2				
118	*S. marcescens*	2	1–2/1	5–6/3–4	4–10/23–26	6–7/55–64
	E. coli ML-35	2				
	GC-F62[c]	2				
	GC-NS[d]	2				
A3.12	*P. putida*	2	1/0	0/0	1/0	1/0
	E. coli ML-35	2				
	GC-F62[c]	2				
	GC-NS[d]	2				
	*GC-miscellaneous (3) (T-1)	1				

[a] All experiments run in both dilute nutrient broth and medium 137 with identical results except *P. mirabilis* versus 109J (137 only), *L. monocytogenes* versus 109J (dilute nutrient broth only), and miscellaneous gonococci (*) versus A3.12 (dilute nutrient broth only). Numbers within braces represent experiments in which no interaction was observed.

[b] Percentage of intact host microorganisms to which bdellovibrio are attached/percentage of host microorganisms that have undergone bdelloplast formation (all data derived from counts of at least 200 target bacterial cells and expressed as the range of values observed).

[c] T1, T3 (gonococcal agar with Isovitalex); T1, T3 (egg passaged); GC: gonococcus.

[d] T1, T2, T3 (gonococcal agar with Isovitalex); T1, T3 (egg passaged).

dality of passaging (culture medium versus egg passaging). Prolongation of experiments up to 7 h failed to provide evidence for gonococcal attack by bdellovibrios.

Although observations in Table 1 are based upon the interaction of bdellovibrios with individual pairs of gonococci, there was never any evidence for bdellovibrio association with gonococci occurring in clumps. Analysis of control data indicated that clumping of gonococci was no more common in the presence than in the absence of bdellovibrios.

The failure of *Bdellovibrio* strain A3.12 to interact with *P. putida*, its normal target, is unexplained. Since this *Bdellovibrio* strain multiplied freely in *P. putida* stock cultures, apparent failure of interaction was presumably a function of the test system used in these experiments.

DISCUSSION

N. gonorrhoeae and the *Enterobacteriaceae* are similar in terms of gram negativity and the chemical and enzymatic composition of their outer membranes (4, 21), but not in their susceptibility to *Bdellovibrio* species. The presence of gonococcal pili or putative surface factors conferred by passaging for virulence were apparently unimportant in the failure of bdellovibrio attack, since nonpiliated, T-3 gonococci passaged through gonococcal agar supplemented with Isovitalex were also unsusceptible to bdellovibrios. Differences in cell membrane composition as reflected by penicillin susceptibility (9) also appeared unrelated since both penicillin-sensitive and -resistant gonococci evaded bdellovibrio invasion.

It is possible that continued screening of gono-

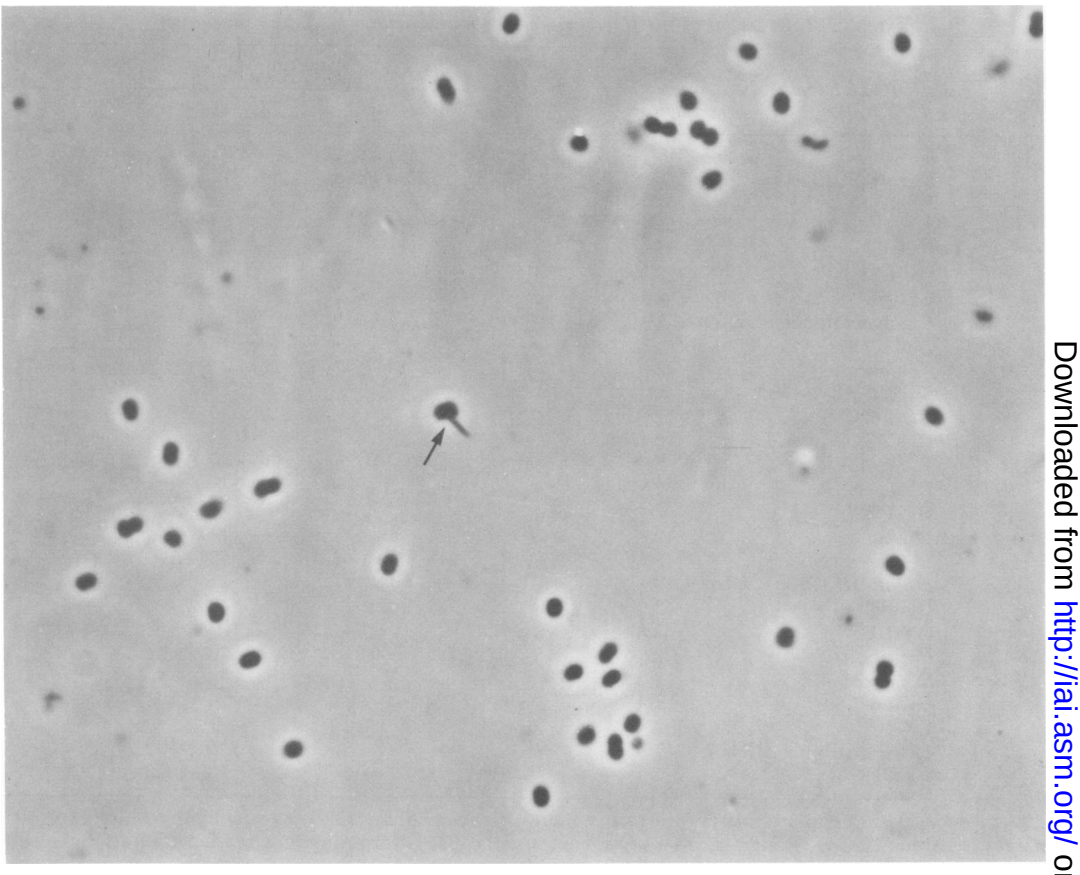

FIG. 1. *Attachment of Bdellovibrio bacteriovorus 109J to egg-passaged F-62 gonococci derived from T-1 colonies. Photograph taken 10 min after mixing of the two microorganisms. Bdellovibrio attachment (arrow) was only transient (phase microscopy; ×2,000).*

coccal isolates will disclose strains that are susceptible to bdellovibrio invasion. It is also possible that an alternative method for demonstrating bdellovibrio-gonococcal interaction might have disclosed an effect upon the gonococci. There are essentially three techniques for evaluating the range of prey susceptible to *Bdellovibrio* species: (i) plaque formation, with use of techniques similar to those employed in bacteriophage experiments, (ii) direct measurement of bdellovibrio-target cell attachment with differential filtration to retain microorganisms to which bdellovibrios have attached, and (iii) lysis of target cell suspensions at high bdellovibrio multiplicities (13). The last alternative was selected because target cell range defined upon this basis is usually broadest (13) and identifies both attachment and bdelloplast formation. Further, the capacity of gonococci to survive on marginally nutritious solid culture medium for the duration required to demonstrate plaque formation was considered questionable.

It is possible that gonococcal-bdellovibrio interaction occurs, but at a level below the sensitivity of the visual test system used in these experiments. This possibility cannot be eliminated without further experimentation; nevertheless, the attachment of bdellovibrios to gram-negative bacilli and the subsequent formation of bdelloplasts could be seen easily by phase microscopy. Therefore, the likelihood of significant undetected gonococcal-bdellovibrio interaction would seem to be minimal. Another possibility that must be considered is that bdellovibrios interact with gonococci, but cannot be seen because of gonococcal clumping in liquid medium. Several lines of evidence argue against this possibility. First, gonococcal clumping was no more common in the presence than in the absence of bdellovibrios. Second, individual gonococcal pairs never showed evidence of bdellovibrio penetration. Third, bdellovibrios were never seen to be associated with gonococci at the periphery of gonococcal clumps. Al-

though it could be argued that bdellovibrios interact selectively with gonococci that occur in clumps, while ignoring those that occur independently, in fact this seems highly unlikely. Therefore, the conclusion that bdellovibrios and gonococci fail to interact under the conditions of these experiments appears sound.

Maeland et al. (7, 8) and Apicella (1) have published data suggesting the presence of gonococcal surface factors that are analogous to the O-somatic antigens of the *Enterobacteriaceae*. Since smooth strains of *E. coli* and *Salmonella* resist bdellovibrio attack, whereas rough strains are more highly susceptible (19), perhaps gonococcal surface factors other than those that were investigated also retard bdellovibrio attachment. It is also noteworthy that the lipopolysaccharides of gonococci apparently contain very low concentrations of heptoses when compared with *E. coli* and *Salmonella* (21). Mutants of *S. typhimurium* whose rough-core polysaccharide lacks all hexoses and heptoses (chemotype Re) have decreased susceptibility to bdellovibrios (19). Whether either of these factors is related to failure of gonococcal attack by bdellovibrios is speculative.

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